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FOREWORD

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INTRODUCTION

The subject of the research in this proposal is the development of methods for the examination of molecular alterations in prostate cancer at the level of single cells. The purpose of the research is to use the developed methods to identify molecular alteration in prostate cancer cells that can be used either singly or in combination to provide insights into the molecular evolution of prostate carcinogenesis, and produce a set of molecular tools capable of influencing the clinical management patients with prostate carcinoma. The scope to the research involves the construction of cDNA libraries representing the genes expressed in selected populations of normal and neoplastic prostate cancer cells followed by the construction of microarrays suitable for comprehensive gene expression studies. These arrays are then used to evaluate methods for single-cell transcriptome amplification with the aim of identifying a cohort of cellular transcripts which correlate with, or 'fingerprint' a cellular phenotype.

BODY

Technical objective 1: To *obtain defined populations of normal and neoplastic prostate cell types which retain in-situ cellular characteristics*

- *Task 1: obtain and pathologically characterize fresh samples of normal, primary neoplastic, and metastatic carcinoma. Prepare tissue sections in frozen and fixed formats. Perform immunohistochemistry.* We have obtained >20 samples of fresh prostate tissue samples representing each of the categories required and have preserved them in frozen and paraffin embedded formats. Immunohistochemistry and interpretation have been performed by our consulting pathologist.
- *Task 2: purify normal luminal, normal basal, and primary carcinoma cell populations using flow cytometric sorting. Disaggregate tissues, immuno-label, sort, assess sorted populations for purity via microscopic examination and by PCR analysis. Sort single cells into microtiter format.* We have sorted and purified normal basal and luminal cells by flow cytometry and constructed a cDNA library from each population. We have sorted primary carcinoma cell populations with variable results in RNA quality and quality. These cells were first microdissected and then sorted by CD44 and CD57 mab. This work is ongoing to optimize the methods.
- *Task 3: evaluate alternative tissue digestion protocols.* We have disaggregated tissue samples with trypsin and with EDTA alone without a significant improvement in quality/quantity of RNA extraction compared to the standard collagenase protocol. Future work will include the use of dispase.
- *Task 4: microdissect cohorts of phenotypically distinct prostate cells: luminal epithelium, basal epithelium, PIN, carcinoma foci, metastatic foci.* We have microdissected cohorts of distinct prostate cancer cells. However, the RNA

yields have not been high due to the tedium of this microdissection approach. Whole transcriptome PCR has produced inconsistent results with indications of degraded RNA starting material (low molecular weight smear by gel electrophoresis). The recent addition of a laser capture microscope will now allow us to evaluate an alternative microdissection approach for rapidity, reproducibility, and sample yield and quality.

- *Task 5: microdissect single cells (20) from each of the above-described phenotypes.* We have been able to successfully microdissect single cells using patch-clamp techniques and deposit them into individual microtiter plate wells. The actual deposition process is verified to the level of visualizing the lack of a cell remaining in the pipette tip, but we do not currently verify that the cell has been deposited into the PCR buffer. We are exploring fluorescence-based methods to follow/track cells for determining the accuracy of cell deposition. The laser capture microscope should also allow for an alternative dissection/deposition method.
- *Task 6: assess RNA quality (preservation) between frozen sections and fixed/stained sections.* We have evaluated 3 samples preserved as frozen sections and 3 samples preserved as fixed embedded preparations. The RNA quality appears to be better in the frozen sections, but the microdissection is more difficult due to cell lysis and DNA/protein viscosity. Dissection from paraffin sections is straightforward, but RNA quality is poor. Future work will examine paraformaldehyde and ethanol fixatives and laser capture microdissection.
- *Task 7: assess feasibility of flow sorted single cell isolation automation.* We have collaborated with Dr. Ger VandenEngh in the Department of Molecular Biotechnology for alternative methods for automated single cell deposition. One approach that will be ready for experimentation in approximately 3 months is a method of cell sorting into wells of linear plastic spooled tape. In addition, we will be experimenting with a cDNA library vector that will sort bacterial transformants containing cDNA inserts based upon size selection.
- *Task 8: (future work) refine cell phenotype acquisition based upon the development of new markers/antibodies.* In collaboration with Dr. Alvin Liu in the Department of Molecular Biotechnology, we have identified several additional antigens recognized with monoclonal antibodies that can be used for sorting prostate epithelial cells by flow cytometry.

Technical objective 2: *To construct microarrays of prostate transcripts that reflect the gene expression potential of the cell types to be examined.*

- *Task 8: identify a non-redundant clone set from the Prostate Expression Database to encompass all highly expressed transcripts (~12), moderately expressed transcripts (~500) and several thousand rare transcripts (~6000).* We have identified a non-redundant set of 3,000 cDNAs (ESTs) from the prostate expression database that are suitable for array construction. Identifying an additional 3,000 cDNAs is in progress.

- *Task 9: retrieve cDNA clones from archive, PCR amplify inserts with amine-linked primer, and purify.* We have retrieved 3,000 cDNA clones from the cDNA archive and amplified the inserts by PCR. Improvements in glass slide coating technology by Amersham has eliminated the need for using amine-linked PCR primers for amplifying clone inserts. We have conducted preliminary experiments using 1500 clone cDNA arrays spotted onto Type 7 mirror-coated Amersham slides. Compared to our previous format, these arrays provide a lower background signal, even spot distribution, higher Cy3 signal, and greater array consistency than our previous format.

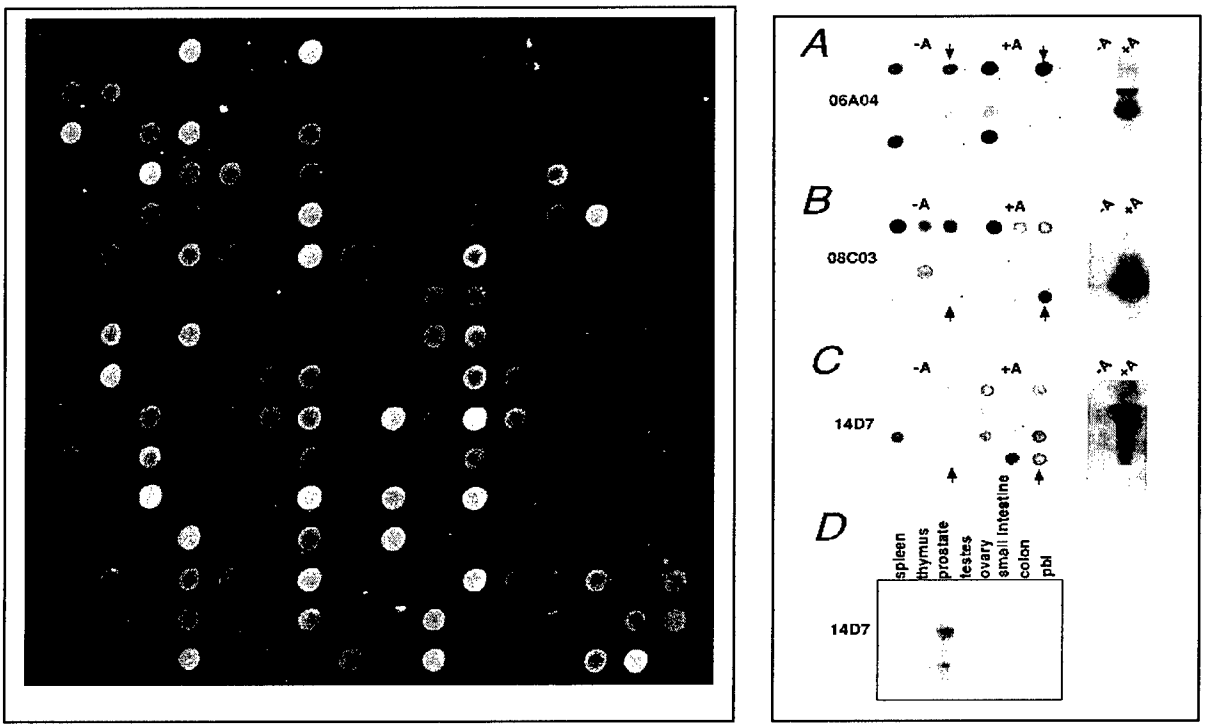

Figure 1A. cDNA microarray. The figure depicts 1/12 of the 1500-cDNA microarray hybridized with total cDNA probes from androgen-stimulated vs androgen-starved prostate cancer cells. Bright spots indicate a gene highly expressed in androgen-stimulated compared to androgen-starved cells. **1B**. Portions of the cDNA microarray containing cDNAs upregulated by androgens with corresponding Northern analysis confirming the results (right panel).

- We have constructed cDNA libraries from flow sorted basal (CD44+), luminal (CD57+), and primary carcinoma (CD44+) cells. The libraries are of good quality with >85% inserts averaging 800bp. Sequencing random clones from each library is in progress to determine the diversity and transcript abundance.
- *Task 11: pick random cDNA clones from the new libraries, array on nylon membranes and screen for abundant prostate cDNAs, select non-abundant species, PCR amplify inserts.* Random cDNA clones from each library have

been picked using the Q-bot picking tool and placed into 384-well microtiter plates. Following the verification of library quality, the clones will be spotted onto nylon membranes for hybridization and negative-selection to acquire low-abundance cDNAs.

- *Task 12: construct physical micro-arrays of cDNA clones on glass supports using robotic tools: total of 500 replicates.* See Task 9 above. We have obtained a Molecular Dynamics Gen III array robot that is capable of >8,000 cDNA clone densities per slide. We will be moving to this format for the prostate microarrays.

- *Task 13: assess alternative array methodologies as they become available (ink jet oligonucleotide)* This task will be on-going for the duration of the proposal.

Technical objective 3: *To construct representative probes from single or small numbers of defined cells that are suitable for micro-array interrogation, and retain the transcriptome composition (diversity and abundance) present in the original cell type(s).* This work is in progress.

Technical objective 4: *To identify a cohort of cellular transcripts which correlate with, define, or "fingerprint", a cellular phenotype(s).*
This work is in progress.

KEY RESEARCH ACCOMPLISHMENTS

- Obtained, pathologically characterized, and archived tissue samples suitable for conducting the proposed research.
- Obtained and purified single circulating neoplastic prostate cells from the peripheral blood of patient with prostate cancer.
- Amplified and sequenced the androgen receptor from a single prostate epithelial cell.
- Constructed cDNA microarrays comprised of 1500 different prostate cDNAs.
- Developed and utilized protocols for array probe construction and hybridization.
- Constructed cDNA libraries from flow-sorted prostate luminal and basal epithelial cells.
- Constructed cDNA library from microdissected prostate luminal and basal epithelial cells.

REPORTABLE OUTCOMES

Nelson PS, Ferguson C, Lin B, White JT, and Hood L. The transcriptome of normal prostate basal and epithelial cells: analysis of shared and divergent gene expression programs. (Manuscript in preparation).

Nelson PS, Ferguson C, Lin B, White JT, and Hood L. The transcript repertoire of single normal and neoplastic prostate epithelial cells. (Manuscript in preparation).

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Nelson PS, Hawkins V, Ferguson C, Vessella R, Lange P, and Hood L. (1999) The Prostate Expression Database: A Tool for Novel Gene Discovery and the Virtual Analysis of Prostate Gene Expression. Proceedings of the American Urological Association. (abstract)

CONCLUSIONS

The research accomplished to date has demonstrated the ability to reproducibly isolate defined prostate cell populations by microdissection and flow cytometry. The isolated cells are of suitable quality for studying genetic alterations at the DNA and RNA level. The research accomplished to date has also produced a tool-set in the form of a cDNA microarray that will now allow for the analysis of the transcript profiles of the defined cell populations. The major problem encountered to date is the inefficiency of our current microdissection methods. Our studies during year 2 of the proposal will involve an evaluation of an alternative microdissection strategy employing a laser capture microscope. The cDNA libraries that we have produced from the defined prostate luminal and basal cell libraries have already been useful as reagents for the cloning of interesting genes that may be involved in the pathogenesis of prostate carcinoma, or that may be used as drug/therapeutic targets. These candidate genes are under further study.

REFERENCES

None

APPENDICES

None